ALCOHOL-INDUCED BREAST CANCER: A PROPOSED MECHANISM

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(Received 1 May 1998; Revised 21 July 1998; Accepted 21 July 1998)

Abstract—Alcohol consumption increases the risk for breast cancer in women by still undefined means. Alcohol metabolism is known to produce reactive oxygen species (ROS), and breast cancer is associated with high levels of hydroxyl radical (•OH) modified DNA, point mutations, single strand nicks, and chromosome rearrangement. Furthermore, ROS modification of DNA can produce the mutations and DNA damage found in breast cancer. Alcohol dehydrogenase (ADH) and xanthine oxidoreductase (XOR) are expressed and regulated in breast tissues and aldehyde oxidase (AOX) may be present as well. Mammary gland XOR is an efficient source of ROS. Recently, hepatic XOR and AOX were found to generate ROS in two ways from alcohol metabolism: by a cetaldehyde consumption and by the intrinsic NADH oxidase activity of both XOR and AOX. The data obtained suggests that: (1) expression of ADH and XOR or AOX in breast tissue provides the enzymes that generate ROS; (2) metabolism of alcohol produces acetaldehyde and NADH that can both be substrates for XOR or AOX and thereby result in ROS formation; and (3) ROS generated by XOR or AOX can induce the carcinogenic mutations and DNA damage found in breast cancer. Accumulation of iron coupled with diminished antioxidant defenses in breast tissue with advancing age provide additional support for this hypothesis because both result in elevated ROS damage that may exacerbate the risk for ROS-induced breast cancer. © 1998 Elsevier Science Inc.

Keywords—Free radical, Xanthine oxidase, Aldehyde oxidase, DNA damage, Breast cancer, Carcinogenesis, Alcohol consumption

INTRODUCTION

Although alcohol consumption has been recognized to increase the incidence of breast cancer in women, no underlying biochemical mechanism has been proposed. We hypothesize herein that reactive oxygen species (ROS) generated from the combined action of alcohol dehydrogenase (ADH) and xanthine oxidoreductase (XOR) mediate alcohol-induced damage to DNA contributing to carcinogenesis and breast cancer.

Alcohol consumption increases the risk for breast cancer

Breast cancer is the result of a complex, multi-stage process [1] in that hereditary susceptibility [2,3], age [3], estrogen metabolism [4–6], tobacco smoke [7] and alcohol consumption constitute recognized risk factors. Recent studies confirm the significant association between alcohol consumption and breast cancer. Analysis of 322,467 women, including 4,335 cases of invasive breast cancer evaluated for up to 11 years, revealed a 41% increase in the incidence of breast cancer by alcohol consumption, and this association was dose dependent up to a dose of 30–60 g alcohol/day [8]. This observation is consistent with several previous studies that revealed an increased risk for breast cancer by alcohol consumption ranging from 20–89% [9–15].

Alcohol metabolism produces ROS

Ethanol can be converted to acetate by a simple, two step reaction involving the combined activities of ADH, that produces acetaldehyde, and the molybdenum hydroxylase enzymes, XOR and/or aldehyde oxidase (AOX), that produce acetate from acetaldehyde. Both
XOR and AOX can generate ROS. Although acetaldehyde also can be metabolized by aldehyde dehydrogenase (ALDH) to produce acetate, this reaction does not form ROS. In the following discussion, we have used the term “reactive oxygen species” to denote three partial reduction products of oxygen: the superoxide anion ($O_2^-$), hydroxyl radical (•OH), and hydrogen peroxide ($H_2O_2$). Metabolism of alcohol has been recognized to produce ROS during acute alcohol toxicity of the liver that has been directly associated with increased ROS damage to DNA leading to DNA modification and strand breakage [16]. Importantly, considerable evidence has been marshaled to indicate that oxidative injury resulting from alcohol toxicity of the liver and pancreas is mediated by ROS generated from the combined activity of XOR and AOX [16–19]. We suggest that a similar process may contribute to the development of breast cancer.

Breast cancer is associated with high levels of •OH modified DNA

DNA from invasive ductal carcinoma of the breast contains extensive •OH modifications that include •OH adducts of adenine, guanine, cytosine, as well as single and double strand breaks [20–22]. •OH modification in breast cancer DNA was elevated between 8 and 17 fold over normal tissue DNA [20] and as many as one base lesion in 46 normal bases has been reported [21]. Furthermore, •OH is of paramount concern for breast carcinogenesis because •OH, but not $O_2^-$ or $H_2O_2$ [23] can modify DNA to produce several •OH adduction products, base deletions, single strand and double strand breaks [20–27]. Importantly, •OH modified DNA has been directly linked to the progression of human breast cancer and provides excellent prognostic information on the progression of breast cancer [22].

Considerable evidence supports a role for alcohol metabolism in carcinogenesis that invokes direct toxicity of acetaldehyde [28–36]. However, acetaldehyde toxicity alone fails to account for the spectrum of DNA alterations found in breast cancer, and in particular, fails to account for the generation of transition and transversion mutations, single and double strand breaks, and •OH modified DNA. We propose that the presence of a highly efficient enzyme system that can further metabolize acetaldehyde may significantly elevate the risk for ROS damage in breast tissues, and these data would be consistent with a reduction in the incidence of breast cancer by diets enriched in antioxidants [37–39].

THE XOR HYPOTHESIS

Our hypothesis is shown schematically in Fig. 1. We propose that: (1) expression of ADH and XOR in breast tissue provides enzymes that generate ROS; (2) metabolism of alcohol produces acetaldehyde and NADH that can both be substrates for XOR and thereby result in ROS formation; and (3) ROS generated by XOR can induce the carcinogenic mutations and DNA damage found in breast cancer tissue. The following discussion provides evidence for each of these points. Furthermore, the role of elevated iron and diminished breast antioxidant status with greater age provide additional support for this hypothesis.

ADH and XOR are expressed in breast tissues

An explicit condition of the present hypothesis is that ADH and XOR or AOX must be present in breast tissues in sufficient abundance to promote ROS formation from alcohol. ADH is expressed in breast tissue. Although most abundant in the liver, ADH activity has been mea-
sured in normal human breast specimens where it was found to yield 23.8 (SD ± 1.6) IU/gram of tissue. ADH levels were not markedly altered by carcinoma when compared to adjacent normal tissue [40].

XOR is also expressed in breast tissue. XOR is derived from the breast by native secretory processes [41] and is routinely prepared from bovine or human milk where it constitutes a large proportion of the total protein mass [42,43]. XOR has been localized by immunohistochemical methods to secretory epithelial cells of the bovine mammary gland [44,45]. Human and mouse mammary epithelial cells in tissue culture also express XOR protein and XOR activity [46–48]. When induced by lactation, XOR comprises between 1–2% of the total mammary gland protein mass [47]. Furthermore, significant levels of XOR are expressed in the non-lactating mammary tissue [47]. Thus, XOR has the potential to comprise a substantial proportion of total breast protein. Although the presence of XOR in breast tissue is also unambiguous, we have been unable to detect substantial levels of AOX activity, protein, or gene expression in mouse mammary glands [49]. Thus, although either XOR or AOX could contribute to ROS burden in the human breast, XOR may be the more important enzyme.

Alcohol consumption may induce both ADH and XOR genes by a common mechanism. ADHs comprise a complex class of genes, ADH1–ADH6, that fall into three enzymatic classes. Class I ADHs, ADH1–ADH3, are most pertinent to alcohol metabolism [50]. Class I ADH genes can be induced at the level of transcription by chronic alcohol administration [51]. Genes encoding XOR are induced 40–80 fold by pregnancy and lactation [47,48]. Regulation of XOR expression may be controlled by STAT factors 3 and 5 that appear to mediate mammary gland development [52,53], and potential STAT factor binding sites can be found in XOR promoters. However, further analysis of XOR promoter sequences also suggests the potential for coordinate regulation with ADH. Transcription factors mediating class I ADH activation include C/EBP, USF, Sp1, NF1, and HNF [50]. Strikingly, promoter sequences for human, rat, and mouse XOR genes also contain recognition sites for C/EBP, USF, Sp1, and HNF [54–57]. These findings support the intriguing possibility that ADH and XOR genes may share some common modes of induction that are possibly related to alcohol consumption.

**XOR can generate ROS in two ways from alcohol metabolism**

Recent biomedical interest in the molybdenum hydroxylase enzymes has revolved around their capacity to generate ROS that have been linked to numerous human diseases [58–60]. Alcohol dependent ROS generation by breast XOR could proceed by two independent mechanisms: metabolism of acetaldehyde and NADH. Thus, it may be of considerable significance that the action of ADH on alcohol stoichiometrically produces NADH. The mechanism of ROS generation by XOR and AOX is now well understood [61,62]. H2O2 and O2•− are generated directly from substrate reduced enzymes when diatomic oxygen acquires reducing equivalents from a reduced flavin site. Rapid, two electron reduction produces H2O2, although kinetically slower univalent reduction produces O2•− [61,62]. 'OH is not a direct product of enzyme catalysis [23,63], however it can be produced secondarily in the Haber-Weiss reaction by interaction with iron or copper in vitro or in vivo [24,64].

**Acetaldehyde metabolism.** XOR can undergo conversion from an NAD+ dependent “D-form” to an oxygen dependent “O-form.” Conversion appears to be a tightly regulated event in the mammary gland as the D-form predominates in the tissue although nearly all of the enzyme in milk is in the O-form [47]. It was long held that ROS generation was a unique property of the O-form. However, recent analysis of D- to O-form conversion indicates that conversion per se is not necessary for ROS generation by XOR [62]. Preparation of XOR from bovine milk entirely in the D-form indicates that the D-form enzyme can react with oxygen to produce ROS at one third the rate of the O-form in the absence of NAD+ [65]. Because D-form XOR has a strong preference for NAD+ over oxygen as an oxidizing substrate, the relative availability of NAD+ or oxygen could become an important determinant of ROS generation by XOR [65]. Although XOR has been defined by its capacity to oxidize xanthine to produce uric acid, XOR can also use numerous other compounds as substrates [61]. Because acetaldehyde is a substrate for both the D- and O-forms of XOR, ROS generation by XOR during acetaldehyde metabolism could be a significant source of ROS in breast tissue.

**NADH oxidase activity of XOR.** The nature of molybdenum hydroxylases in human breast tissue is complicated by reports that XOR in human milk is catalytically inactive toward conventional substrates [43,66]. However, the molybdenum hydroxylase enzymes, AOX and XOR, also possess kinetically efficient intrinsic NADH oxidase activities [17,23,65,66]. XOR has a Km for NADH of 2.8 μM, in contrast to its Km for acetaldehyde that has been reported to be from 100 μM to 16 mM depending on the source [61]. Also, the catalytic rate constant for NADH oxidation by XOR in the presence of oxygen is comparable to that for xanthine utilization and reflects comparably efficient Vmax for both substrates [65,66]. Thus, NADH would be a more efficient sub-
strate for XOR than would acetaldehyde in tissues expressing this enzyme. The NADH oxidase activity of both molybdenum hydroxylase enzymes also generates ROS [17,23,65,66]. Moreover, the ROS injury sustained during alcohol toxicity of the liver appears to operate entirely through the combined NADH oxidase activities of XOR and AOX and injury appears to be mediated by an iron dependent process [16–18]. Although human XOR possesses low catalytic activity toward purine substrates it has efficient NADH oxidase activity [43,65,66]. We suggest that NADH may be an important, but largely overlooked, substrate for XOR in the breast and that the intrinsic NADH oxidase activity of XOR may also generate ROS in the breast.

**ROS modification of DNA can produce the mutations found in breast cancer**

In the absence of contaminating metals, DNA is resistant to H$_2$O$_2$ to a concentration of at least 10 mM [23]. Addition of only picograms of ferrous iron is sufficient to convert H$_2$O$_2$ into •OH and induce DNA modification and single strand cleavage [24,26,64]. Thus, •OH is considered to be the ROS species that induces DNA modification and strand breakage. Although singlet oxygen (O$_2^\cdot$), an energetically activated form of diatomic oxygen, can also induce the modifications considered here, it probably has little relevance to alcohol toxicity [67,68] and is not produced by the molybdenum hydroxylase enzymes.

Hydroxyl radical induces numerous modifications to DNA [20,23–27] including single base deletions, base ring opening (largely adenine and guanine) [21] single and double strand breakage, and adduction of bases to form chiefly 5-OH-cytosine, 8-OH-guanine, and 8-OH-adenine, although many other sites of •OH attack have been identified [69]. Single strand breakage, for example, would be estimated to represent no more than 10% of the hydroxylation events [20,26,27,69]. Thus, no single measure of oxidative DNA damage fully represents the extent of DNA modification.

Metabolically derived oxygen-based ROS can be mutagenic [70]. However, not all of the hydroxylation events on DNA have proved to be mutagenic. For example, base ring opening has not been linked to mutagenesis, and single or double strand cleavage may be more involved in chromosome deletion and rearrangement. Interest has centered, however, on 8-OH-deoxyguanosine (8-OH-dG) as an indicator of oxidatively modified DNA that can be mutagenic [68,71]. 8-OH-dG is a common and easily measured modification that is markedly elevated in breast cancer [20–22], G:C to T:A transversion mutations have been specifically linked to 8-OH-dG in post replication cells [68,71], and current focus on 8-OH-dG reflects its great abundance in oxidatively modified DNA. 5-OH-deoxy cytosine can also be mutagenic, producing C::G to T::A transition mutations [72].

Oxidative DNA modification is most likely random, and specific genetic targets for ROS generated mutations in carcinogenesis of the breast have not been characterized. However, a number of somatic mutations have been identified in breast cancer [73]. These include transversion mutations of p53, BRCA1, and BRCA2. These key genes have been linked to breast cancer progression [74–77] and the mutations found in them can be produced by ROS [74]. Although the direct link between alcohol-induced ROS modification of DNA and mutation of these genes remains to be established, they should be considered important candidates for alcohol-induced carcinogenesis because mutations in these genes could be responsible for tumor initiation as well as tumor progression.

**FURTHER SUPPORT FOR THE XOR MEDIATED ROS HYPOTHESIS OF BREAST CANCER**

Iron accumulation may potentiate ROS damage to breast DNA

Iron can catalyze conversion of H$_2$O$_2$ into •OH, the primary ROS responsible for damage to DNA. Iron has been recognized to potentiate carcinogenesis in several different organ systems and is an important risk factor for breast cancer [78,79,80]. For reasons not fully understood, iron accumulates in intracellular complexes with ferritin storage protein as a function of age. Thus, males and females reveal progressive iron accumulation with age that is especially enhanced in post-menopausal women [81] whose incidence of breast cancer is increased.

Importantly, serum and breast ferritin levels are substantially elevated in breast carcinoma [82,83] and have been directly linked to mammary carcinogenesis through ROS [83]. It has been suggested that elevated ferritin/iron complexes may supply the unusual needs for iron during proliferation of mammary tissue in normal or breast carcinoma cells. However, the need for iron in mammary gland cell growth may also substantially contribute to an unusual risk for ROS mediated injury.

**Diminished antioxidant defenses in breast tissue may enhance ROS damage to DNA**

All cells are subjected to a constant barrage of metabolically derived ROS either from enzyme sources, of which the molybdenum hydroxylases are only one example, or from mitochondrial electron transport [84].
Intracellular defenses against this oxidative onslaught are numerous with a primary role attributable to the enzymes superoxide dismutase (SOD), catalase (CAT), and the glutathione (GSH) redox system [85]. The presence of both efficient ROS scavenging systems and ROS generating enzymes has enabled the view of an oxidant/antioxidant balance composed of these opposing elements. Disruption of this balance by excessive ROS generation or diminished ROS scavenging capacity will predispose the cell to oxidative injury.

Levels of both SOD and CAT in mammary glands from rodents and humans decline with age [86,87,88], as do the levels of GSH in human mammary glands [89,90]. Furthermore, in mammary carcinoma tissues from humans and mice CAT levels are even more markedly diminished [86,87]. The combination of age associated diminished ROS scavenging and a potent mechanism for ROS generation could promote excessive ROS injury of the breast. We would argue that diminishing levels of catalase with age in conjunction with naturally elevated iron stores places mammary cells at unusual risk for alcohol-induced ROS damage to DNA contributing to mammary carcinogenesis.

**DISCUSSION**

We have proposed an explicit model for alcohol-induced ROS generation that depends on the combined activities of ADH and XOR. The direct action of cytochrome p450 2E1 on ethanol in the mammary gland may be an additional source of carcinogenic ROS [91,92]. Although the role of ROS in carcinogenesis is still being defined, the amelioration of several cancers, including breast cancer, by antioxidants underscores the importance of confirming this mechanism. Alcohol derived ROS could contribute to several stages in breast cancer development. For example, alcohol derived ROS could act at an early stage of mutagenesis leading to tumor initiation and breast cancer, at later stages of progression and transformation to a cancer phenotype, or perhaps affect cell proliferation. We have focused on the role played by ROS in DNA modification and carcinogenesis. However, potential effects on proteins such as the redox sensitive transcription factors AP-1 and NF-κB may also play important roles in transformation.

Improved understanding of the causes of breast cancer could lead to improved diagnosis and treatment. The realization that ROS may participate in the development of breast cancer suggests that removing potentially carcinogenic ROS may have therapeutic value. Treatment with ROS scavenging antioxidants, inhibition of critical ROS generating enzymes like XOR, reduction of iron intake, and reduced alcohol consumption by women in the higher age dependent risk categories could significantly modulate the incidence of breast cancer.

**Acknowledgements** — Supported by grants from the National Institutes of Health (HL52509 and HL45582), The Muscular Dystrophy Association, and The Robert and Helen Kleberg Foundation.

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ABBREVIATIONS

ADH—alcohol dehydrogenase
AOX—aldehyde oxidase
GSH—glutathione
H₂O₂—hydrogen peroxide
O₂⁻—superoxide anion
•OH—hydroxyl radical
XOR—xanthine oxidoreductase